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Title

Injectable diblock copolypeptide hydrogel provides platform to maintain high local concentrations of taxol and local tumor control

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Abstract

The current treatment of glioblastoma multiforme (GBM) is limited by the restricted arsenal of agents which effectively cross the blood brain barrier (BBB). For example, only a fraction of Temozolamide (TMZ) administered systemically is available for therapeutic effect because of the BBB and the instability of TMZ under physiologic conditions. A novel approach to overcome this obstacle is to bypass the BBB and locally deliver chemotherapeutic agents directly to the tumor mass. We have explored the loading of TMZ into a novel hydrogel matrix, which can be delivered in liquid form and then solidifies in situ and releases chemotherapy as the matrix dissolves. Here, we tested the effect of amphiphilic diblock copolypeptide hydrogels (DCHs) of 180-poly-lysine and 20-poly-leucine (K180L20) on TMZ using Glioblastoma models. In both the in vitro model, which involved treatment of a human glioblastoma GSC line suspended as neurospheres, and in vivo using an orthotopic glioma xenograft mouse model, we found that K180L20 could safely enhance the efficacy of TMZ. This technique may offer the opportunity to 'coat' the inner lining of the cavity following glioma resection with a slow-release TMZ and potentially decrease recurrence. Future studies in larger animals are needed to delineate this effect.

Keywords	hydrogel; TMZ; glioma
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Dear Editors of *Journal of Clinical Neuroscience*,

Enclosed please find a manuscript entitled “The use of TMZ embedded hydrogels for the treatment of orthotopic human glioma xenografts.” We are submitting this manuscript as an original research article to *Journal of Clinical Neuroscience*.

In this manuscript, we report a novel approach to overcoming the challenge posed by the blood brain barrier in the treatment of glioma with chemotherapy. We found that by loading TMZ into a hydrogel matrix, the efficacy of TMZ against glioblastoma was enhanced. This was consistent between in vitro and in vivo animal models, with low cytotoxicity. We believe this technique may offer the opportunity to coat the inner lining of the glioma resection cavity and potentially decrease the rate of tumor recurrence when applied to larger animals.

We believe that these findings are of interest to the clinical neuroscience community and that the manuscript describing this work would be of interest to your readership. Thank you for your consideration.

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The use of TMZ embedded hydrogels for the treatment of orthotopic human glioma xenografts

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Abstract

The current treatment of glioblastoma multiforme (GBM) is limited by the restricted arsenal of agents which effectively cross the blood brain barrier (BBB). For example, only a fraction of Temozolamide (TMZ) administered systemically is available for therapeutic effect because of the BBB and the instability of TMZ under physiologic conditions. A novel approach to overcome this obstacle is to bypass the BBB and locally deliver chemotherapeutic agents directly to the tumor mass. We have explored the loading of TMZ into a novel hydrogel matrix, which can be delivered in liquid form and then solidifies in situ and releases chemotherapy as the matrix dissolves. Here, we tested the effect of amphiphilic diblock copolypeptide hydrogels (DCHs) of 180-poly-lysine and 20-poly-leucine (K₁₈₀L₂₀) on TMZ using Glioblastoma models. In both the in vitro model, which involved treatment of a human glioblastoma GSC line suspended as neurospheres, and in vivo using an orthotopic glioma xenograft mouse model, we found that K₁₈₀L₂₀ could safely enhance the efficacy of TMZ. This technique may offer the opportunity to 'coat' the inner lining of the cavity following glioma resection with a slow-release TMZ and potentially decrease recurrence. Future studies in larger animals are needed to delineate this effect.

Keywords: hydrogel; TMZ; glioma

Introduction

Glioblastoma multiforme (GBM) is the most common and most aggressive primary malignant brain tumor [1, 2]. The current standard treatment is surgical resection, followed by adjuvant radio- and chemo-therapy [1-3]. However, the median survival is only approximately 15 months [1-3]. The failure of cancer therapy is multifactorial including a genetically evolving tumor that escapes initial therapy and the restricted arsenal of agents which effectively cross the blood brain barrier (BBB) [4-6].

Temozolomide (TMZ) chemotherapy has been a significant advance in the chemotherapy of GBM [3, 6]. TMZ is administered systemically (oral or intravenous); however, only a fraction of total administered drug is available for therapeutic effect because of the BBB and the instability of TMZ under physiologic conditions [6, 7]. In brief, TMZ crosses the BBB into the brain cells, hydrolyses into DNA alkylating agent 5-(3-methyl-triazene-1-yl)imidazole-4-carboxamide (MTIC) and causes cytotoxic damage to DNA [8]. TMZ can also degrade to MTIC in the bloodstream, which is problematic for treatment as MTIC does not cross the BBB and may cause hematologic toxicity [7]. Due to short half-life and poor penetration through the BBB, the patients must be administered with high doses of TMZ for a long period, which can lead to serious systemic toxicity [3, 6].

To overcome this obstacle, a novel approach is to bypass the BBB and locally deliver chemotherapeutic agents directly to the tumor mass. As an alternative approach to systemic delivery of TMZ, in the current report we have explored the loading of TMZ into a novel hydrogel matrix, which can be delivered in liquid form and then solidifies in situ and releases chemotherapy as the matrix dissolves [9, 10]. This strategy was chosen based on reports that amphiphilic diblock copolypeptide hydrogels (DCHs) are capable of releasing chemotherapeutic agents in a rate-dependent manner by loading them in the matrix [9, 10]. Among these hydrogels, DCHs of 180-poly-lysine and 20-poly-leucine ($K_{180}L_{20}$) have been shown to have low cytotoxicity, controlled release of loaded drugs and biodegradability [9, 10]. Here, we tested the effect of $K_{180}L_{20}$ on TMZ using Glioblastoma models and the result indicated that $K_{180}L_{20}$ could enhance the efficacy of TMZ significantly both in vitro and in vivo.

Results

$K_{180}L_{20}$ is not toxic, in vitro and in vivo

To examine the toxicity of $K_{180}L_{20}$, NHA cells and GBM001 neurospheres (NSs) were treated in cell culture with Vehicle (PBS), TMZ (100 μ M), $K_{180}L_{20}$ (0.01%) or $K_{180}L_{20}$ (0.01%)/TMZ (100 μ M), separately (Fig. 1A).

In both cell types, K₁₈₀L₂₀ alone did not show significant cytotoxicity. However, in NS cells, K₁₈₀L₂₀/TMZ decreased the viability from 60% to 20% compared to TMZ treatment alone, indicating that K₁₈₀L₂₀ might enhance the efficacy of TMZ specifically in tumor cells. Furthermore, orthotopic implantation of 4 μ L of K₁₈₀L₂₀ (0.5%) with or without TMZ (50mM) treatment into Nu/Nu mice did not cause notable cell death detected by H&E staining (Fig. 1 B), or significant inflammation response by CD11b staining (Fig. 1C).

K₁₈₀L₂₀ enhances the efficacy of TMZ

Next, we investigated the role of K₁₈₀L₂₀ in the efficacy of TMZ in vivo using an orthotopic glioma xenograft mouse model. GBM001 cells stably transduced with GFP/Luc were implanted into Nu/Nu mice, followed by the treatment with 4 μ L of vehicle (PBS), K₁₈₀L₂₀ (0.5%), TMZ (50mM) or K₁₈₀L₂₀ (0.5%)/TMZ (50mM), separately. 11 days post treatment, IVIS BLI images were taken to evaluate the tumor size (Fig. 2A). Consistent with in vitro result, though tumors in K₁₈₀L₂₀ group were comparable to the ones in vehicle group, combination of K₁₈₀L₂₀ and TMZ dramatically decreased tumor size compared treatment with TMZ alone. Moreover, H&E staining showed significant shrinkage of tumor in the K₁₈₀L₂₀/TMZ group (Fig. 2B), indicating that K₁₈₀L₂₀ could enhance the efficacy of TMZ in vivo.

Finally, the median survival of mice implanted with GBM001 was 20 days in both vehicle and K₁₈₀L₂₀ groups, which was prolonged to 28 days by TMZ treatment (Fig. 3). Combination of K₁₈₀L₂₀ and TMZ extended the median survival to 38 days, significantly (p=0.0285) (Fig. 3).

Materials and Methods

Materials

Normal Human Astrocytes (NHA) were purchased from ScienCell, and GBM001 was established as a glioblastoma GSC line derived from a patient specimen and underwent <10 passages before use. Both cell lines were passaged at 37°C and 5%CO₂. NHA was cultured in Astrocyte medium (ScienCell) supplemented with FBS, astrocyte growth supplement and penicillin/streptomycin. GBM001 was maintained as tumor spheres in neurobasal medium (DMEM F12/Glutamax, Gibco) supplemented with B-27, Glutamax, 0.2% Heparin, EGF and FGF according to the manufacturer's instruction. GFP/Luc Lentiviral construct was generously provided by Dr. Dong-Er Zhang (University of California, San Diego). Temozolomide (TMZ) was purchased from AK Scientific and freshly prepared for experimental use. D-luciferin firefly (Xenogen, XR-1001) was

purchased from PerkinElmer. Peptide based hydrogel Lysine180 leucine 20 ($K_{180}L_{20}$) was provided by Amicrobe, Inc.

Drug Formulation

$K_{180}L_{20}$ was dissolved in PBS at 1% overnight at 37°C and vortexed rigorously to ensure a homogenous solution. Final concentration of $K_{180}L_{20}$ was 0.01% for the experiments in vitro and 0.5% for in vivo.

Transduction

GFP/Luc lentiviral particle was packaged using previously described methods [11]. GBM001 cells were transduced by GFP/Luc lentivirus for 3 days. Stable cells were generated by flow cytometric sorting using GFP.

Cell Viability Assay

Cells were plated in 96-well plates followed by the treatment with Vehicle (PBS), $K_{180}L_{20}$ (0.01%), TMZ (100 μ M) or $K_{180}L_{20}$ (0.01%)/TMZ (100 μ M) formulation, separately. 5 days post-treatment, cellular viability was determined by CellTiter-Blue assay (Promega).

Orthotopic xenograft model

All animal procedures were performed in accordance with the requirements and recommendations in the Guide for the Care and the Use of Laboratory Animals approved by UCSD Institutional Animal Care and Use Committee (IACUC) and National Institute of Health (NIH).

5-6 weeks old Nu/Nu mice were injected orthotopically by 2×10^4 GBM001 cells transduced with GFP/Luc. The coordinates were 1.8 mm to the right of bregma and 3 mm deep from the dura. 7 days post injection, mice were stereotactically injected with 4 μ l of vehicle, $K_{180}L_{20}$ (0.5%), TMZ (50mM) or $K_{180}L_{20}$ (0.5%)/TMZ (50mM) formulation, separately. Mice with >20% reductions in body weight were sacrificed, and brains were processed for paraffin or frozen section.

IVIS Imaging

Bioluminescent images (BLI) were taken at 11 days post-treatment under Xenogen IVIS 200. D-luciferin (150mg/kg body weight) was administered into the mice via intraperitoneal (IP) injections and allowed to react for 10 minutes before imaging. Animals were then anesthetized with 2.5% Isoflurane and imaged under CCD camera; exposure time 0.2 sec, binning 8, field of view E (26.5 cm), f/stop (1), and emission filter

open. Signal was measured and recorded as total flux (photons/sec). The results were then analyzed using Living Image Software (3.0).

H&E and Immunohistochemical (IHC) staining

Mice brains were harvested at 11 days post-treatment, followed by fixation using 4% PFA. The brains then were embedded in paraffin and sectioned in the UCSD Histology core. The slides were processed for H&E and IHC staining, separately. For IHC staining, the slides were stained with rabbit anti-CD11b antibody (1:200, Abcam), followed by anti-rabbit alkaline phosphatase secondary antibody (Vector Laboratories).

Discussion

In the present study, we demonstrated that K₁₈₀L₂₀ could enhance TMZ efficacy both in vitro and in vivo in glioblastoma. Given the characteristics of diblock copolypeptide hydrogels (DCH) to deliver hydrophobic molecules directly into the brain and control the molecular release, we hypothesized that DCH might influence the efficacy of the loaded hydrophobic drug [9]. We loaded TMZ as the hydrophobic drug into the K₁₈₀L₂₀ DCH polymer and performed in vitro studies on Astrocytes and tumor cells, and in vivo study in nude mice. Both in vitro and in vivo studies showed minimal or no toxicity and enhanced efficacy of TMZ with K₁₈₀L₂₀. These observations suggest that K₁₈₀L₂₀ is innocuous and does indeed enhance the efficacy of TMZ. The enhancement in the efficacy of TMZ, based on previous findings, can be attributed to drug release kinetics of DCHs. DCHs can release loaded hydrophobic drugs with a certain release profile, and the kinetics of release depend upon the qualitative and quantitative formulations of the drug and polymer [9]. Prior studies by T. Deming et al. have shown that higher concentrations of DCH prolonged the drug release profile; 4% K₁₈₀L₂₀ showed the most prolonged drug release profile followed by 3% and then by 2% in in-vitro studies [9]. In our study, only 0.5% K₁₈₀L₂₀ was enough to bring about the change in the in vivo study with a median survival of 38 days in K₁₈₀L₂₀/TMZ group versus 28 days in TMZ group. We believe using a higher concentration of K₁₈₀L₂₀ could have prolonged the median survival, but limitations of DCH in solubility and injectability play a vital role in executing such studies. One of the problems noted during our study was that the DCH polymer provided by Amicrobe, Inc. was not dissolvable at 3% by volume in water. Also, the dissolved hydrogel polymer tends to solidify at room temperature; therefore, maintaining the hydrogel/drug formulation at 37°C at all times during the experiment might be a useful approach. In terms of solubility of DCHs, an easier and readily dissolvable method for K₁₈₀L₂₀ DCHs needs to be developed in order to be able to inject higher concentrations of DCH for more sustained drug release.

Following resection of human glioma (either total or subtotal), a resection cavity is present. Prior studies have shown that typically, initial recurrence of glioma occurs within 2 cm of this resection cavity (12). This has been the rationale behind the development of chemotherapy embedded wafers which are placed in the resection cavity following surgery in place of or to enhance the efficacy of systemically delivered chemotherapy. The K₁₈₀L₂₀ DCHs offer interesting alternatives in this regard in that the polymer is initially extruded into the resection cavity in a liquid form and then solidifies. This offers the opportunity to have a more conformal chemotherapy embedded polymer, with the ability to 'coat' the inner lining of the resection cavity with a slow-release TMZ. Future studies in larger animals will better delineate the performance characteristics for recurrence prevention of larger volumes of the polymer in the context of glioma resection.

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Figure Captions:

Fig 1. Toxicity of K₁₈₀L₂₀ in Normal Human Astrocytes (NHA) and GBM001 neurospheres. (A) CellTiter-Blue Survival Assay. NHA and GBM001 neurospheres (2×10^3 cells per well) were treated with K₁₈₀L₂₀ (0.01%), TMZ (100 μ M), or K₁₈₀L₂₀ (0.01%)/ TMZ (100 μ M), and cellular viability was measured 5 days after the treatment. (B) H&E Staining. Nu/Nu mice were intracranially injected with K₁₈₀L₂₀ (0.5%) or K₁₈₀L₂₀ (0.5%)/TMZ (50 μ M) and sacrificed 1 day post injection. (C) Immunohistochemical (IHC) staining. The brain sections from (B) were stained with anti-CD11b antibody (1:200, Abcam), followed by anti-rabbit alkaline phosphatase secondary antibody (Vector Laboratories).

Fig 2. K₁₈₀L₂₀ enhances the efficacy of TMZ in vivo. (A) Representative bioluminescent Images. 2×10^4 GFP/Luc transduced GBM001 neurospheres were implanted into Nu/Nu mice stereotactically. 7 days post-tumor-implantation, animals were treated with Vehicle (PBS), TMZ (50mM), K₁₈₀L₂₀ (0.5%) or K₁₈₀L₂₀ (0.5%)/TMZ (50 μ M), separately. IVIS images were taken 11 days post-treatment and mice were sacrificed for IHC staining. (B) H&E staining. The brain sections from (A) showed variable tumor size among 4 different treatment groups.

Fig 3. Kaplan-Meier Survival Curve. Nu/nu mice were implanted intracranially as described in Fig 2. 7 days post-implantation, mice were treated with vehicle (n=12), TMZ (50mM) (n=12), K₁₈₀L₂₀ (0.5%) (n=12) or K₁₈₀L₂₀ (0.5%)/TMZ (50 μ M) (n=11), separately. The percentage of surviving mice (y-axis) is plotted with respect to time in days (x-axis). Note: p-value of TMZ (50mM) vs. K₁₈₀L₂₀ (0.5%) /TMZ(50 μ M) is 0.0285.

Figure 1. Toxicity of K₁₈₀L₂₀ and TMZ in vitro and in vivo

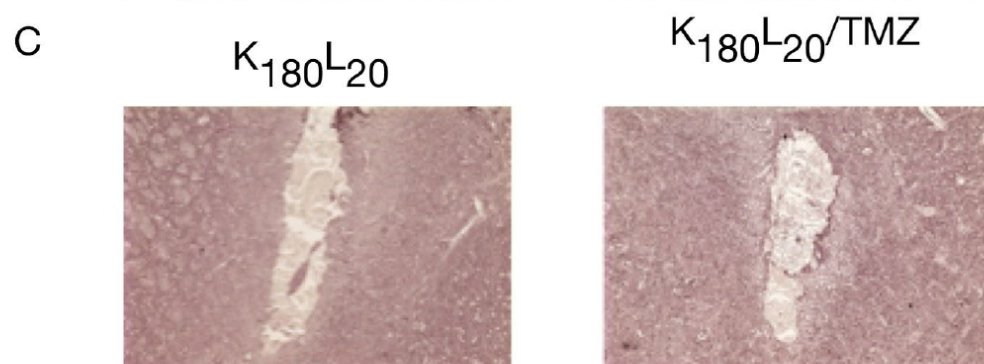
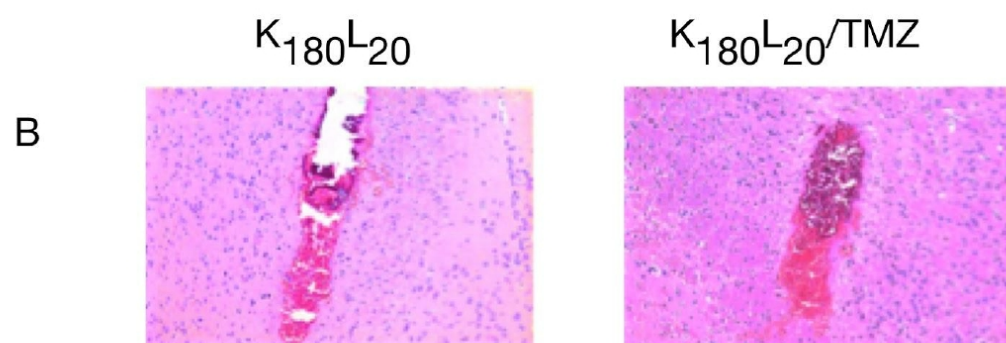
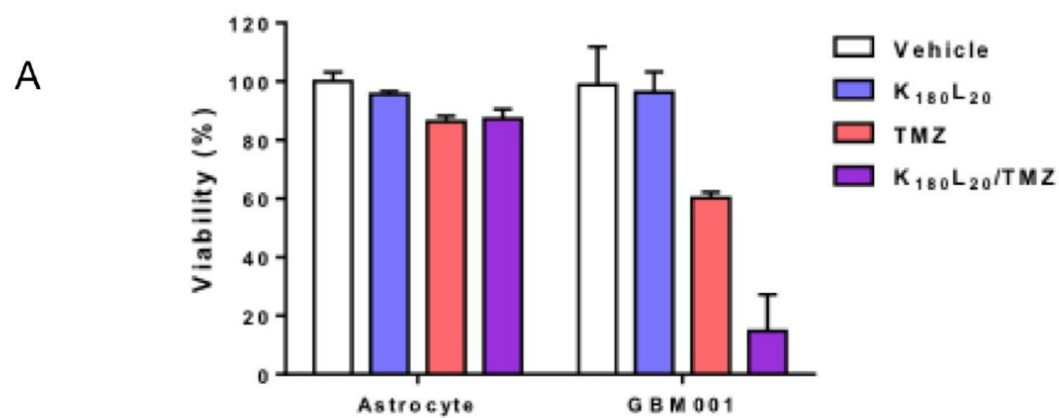


Fig. 2. $K_{180}L_{20}$ enhances the efficacy of TMZ

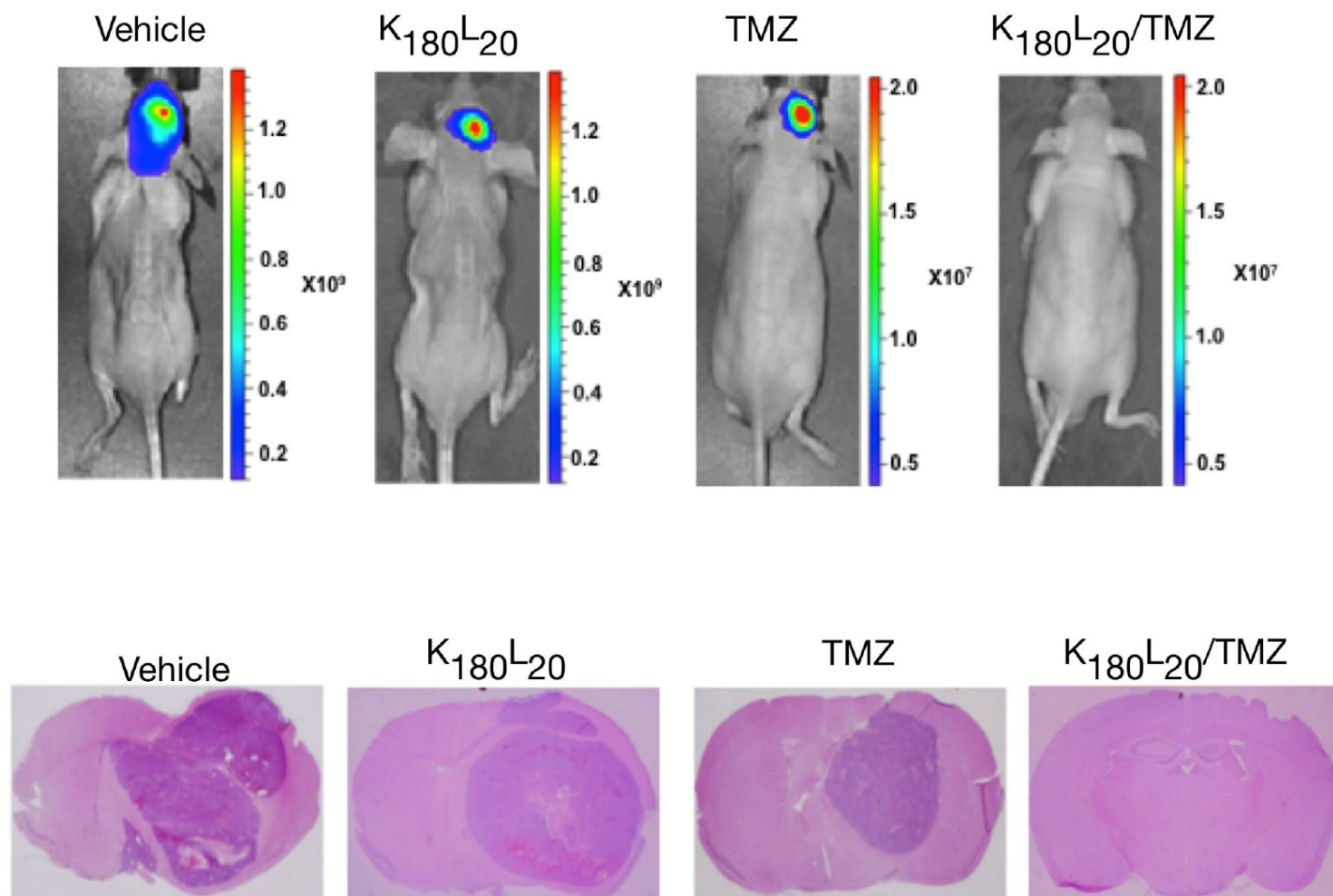
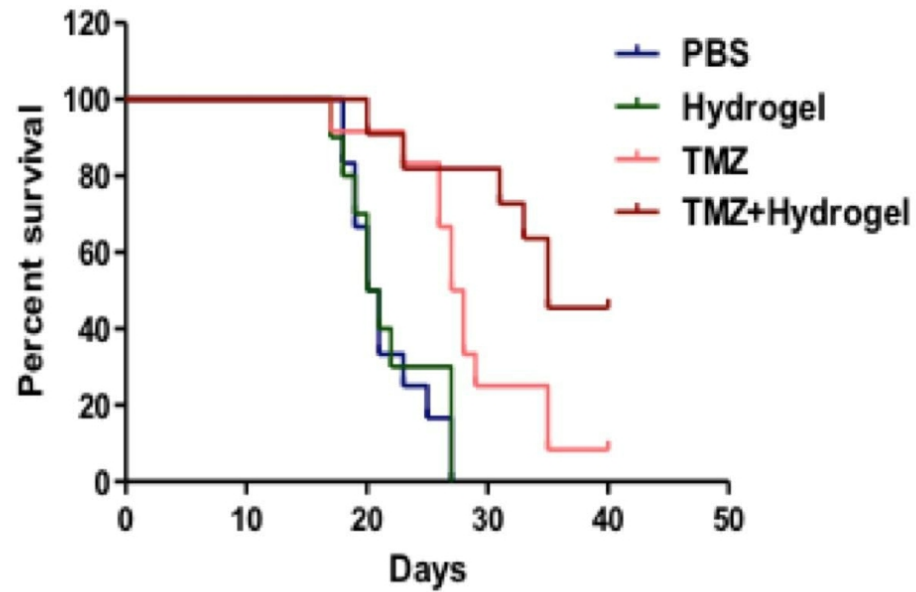


Figure 3. Kaplan-Meier Survival Curve



Groups	P-Value
Vehicle vs. K ₁₈₀ L ₂₀	0.7028
Vehicle vs. TMZ	0.0009
Vehicle vs. K ₁₈₀ L ₂₀ /TMZ	<0.0001
K ₁₈₀ L ₂₀ vs. TMZ	0.0047
K ₁₈₀ L ₂₀ vs. K ₁₈₀ L ₂₀ /TMZ	0.0002
TMZ vs. K ₁₈₀ L ₂₀ /TMZ	0.0285

Highlights

- Therapeutic effect of systemic Temozolomide is limited by the blood brain barrier.
- Temozolomide can be loaded onto a novel hydrogel matrix.
- Hydrogels locally deliver TMZ to the tumor mass and bypass the BBB.
- The hydrogels tested are not toxic in vitro or in vivo.
- The efficacy of TMZ in glioblastoma models is enhanced by hydrogel delivery.

The use of TMZ embedded hydrogels for the treatment of orthotopic human glioma xenografts

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Conflicts of interest: none.